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13. ABSTRACT *(Maximum 200*
The purpose of this research project is to assess if PhIP exposure at dietary levels presents a human breast cancer risk. During the period July 15, 1994-September 30, 1998 we have studied the pharmacokinetics of PhIP following acute oral and chronic administrations at dietary-relevant doses. We have shown that PhIP is bioavailable to the breast and forms DNA adducts in this tissue following oral administrations. PhIP also forms adducts with the blood proteins albumin and hemoglobin. We have been able to demonstrate dose-response trends for tissue concentrations and adduct levels of PhIP in liver, colon and breast tissue in both male and female F344 rats, indicating that even at low dose, PhIP exposure may present a cancer risk. In chronic studies we have shown that PhIP accumulates in the tissues and that excretion occurs mainly through the feces. In lactating rats we have shown that PhIP is passed from the milk to suckling pups and may pose a carcinogenic risk to the pups. We have established that the majority of DNA adducts formed are with deoxyguanosine and the major adduct is dG-C8 PhIP. Furthermore, we have produced antibodies against PhIP-DNA and have developed a fluoroimmunoassy to detect and quantify PhIP-DNA adducts for use in molecular epidemiology studies. This work was performed under the auspices of the US Department of Energy under contract W-7405-ENG-48.

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FOREWORD

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Ken Whitton 9/1/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION
SUBJECT AND BACKGROUND OF PREVIOUS WORK

A great deal of concern has been expressed that cooking meat produces genotoxic substances which may contribute to the incidence of human cancers. Of all the substances known to be produced during cooking, the most important may be a class of heterocyclic amines called the imidazoazaarenes (AIA). These heterocyclic amines are considered to be significant because they are produced at relatively low cooking temperatures such as occur through the grilling, frying, and broiling of red meats, poultry, fish, and grain (1-3). Several of these compounds have also been found in beer and wine and in cigarette smoke condensates (4-6). The AIA currently identified from cooked foods consist of 19 compounds classified generally as quinolines, quinoxalines, phenylpyridines, and carbolines. All quinoline, quinoxaline and carboline AIAs characterized to date are very potent *Salmonella* mutagens (>100,000 rev/mg). 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a phenylpyridine, is a relatively weak *Salmonella* AIA heterocyclic amine mutagen (2,000 rev/mg), but is the most potent in Chinese hamster ovary cell (CHO) genotoxicity assays (7-9). Other important food-borne carcinogens, such as aflatoxin B_1 or benzo[a]pyrene, are orders of magnitude less potent in genotoxicity assays than the AIAs (10). Importantly, of the 19 known food-borne AIAs, 10 have been tested for carcinogenicity and all ten have been found to induce tumors in both rats and mice; and in multiple organs (2,11,12). Of the AIA identified, we considered PhIP to be most important since it is present in the highest concentration in well-done beef (2), has been found in cooked grains, beer, wine, and in cigarette smoke; and, unlike most heterocyclic amines, causes breast tumors in the rat (13). Of equal importance, the human exposure of PhIP has been documented as PhIP has been detected in human urine after consumption of normal diets (14,15). Given the recent findings that mutations in the *p53* gene of breast cancer patients are more similar to mutations caused by chemical mutagens than to spontaneous mutations, the role of compounds like PhIP in the etiology of human breast cancer should be critically evaluated (16).

Non-human genotoxicity & metabolism

The mechanism of PhIP's genotoxicity has been most adequately characterized in rodents, but several studies have been carried out in non human primates and human tissue fractions. Understanding these mechanisms is critical to determining if PhIP can cause breast cancer in humans and how to predict an individual's susceptibility. Further, understanding these mechanisms is important since species and tissue specificity in metabolism can ultimately affect the extrapolation of the animal data to humans. PhIP is excreted via the urine and feces, and several stable and unstable DNA- and protein-reactive metabolites have been measured and identified (20-25), although pathways may be dose dependent (26). Pharmacokinetics, metabolism, clastogenicity, and DNA adduct formation have also been measured for PhIP, albeit at exposure levels orders of magnitude greater than found naturally and for tissues other than breast (27-36). Some data have been reported in non-human primates (37-40). The sum of the bioassay data shows conclusively that PhIP is a potent genotoxin and carcinogen. The mutagenicity, and presumably the carcinogenicity of PhIP results from metabolic activation of the parent heterocyclic amine. This principally results from oxidation of the exocyclic amino group to its corresponding N-hydroxylated derivative (2-N-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine) by the cytochromes P450 (26, 41, 42). The initial

oxidation of the PhIP molecule by the cytochromes P450 is followed by one of several conjugations of the exocyclic N-hydroxyl group with acetate, sulfate or other constituents (43-45). Interspecies differences in metabolism have been suggested since rabbit P450IA1 is more active with PhIP than the corresponding P450IA2, whereas human, rat, and mouse P450IA2 is more active than the corresponding P450IA1 (28,46,47). Additionally, N-hydroxy-PhIP is preferentially sulfated in mice (44) and preferentially acetylated in human tissue fractions (Turteltaub *et al.*, unpublished). Such interspecies differences in metabolism may be significant for risk assessment and needs to be understood prior to assessing PhIP's role in human breast cancer. Likewise, the role of the breast in generating bioactive intermediates needs to be understood to develop markers for susceptibility and to understand what makes the breast a target for chemical agents like PhIP.

The principal detoxification pathway for PhIP in rodents and non human primates involves hydroxylation at the 4'-position of the phenyl ring by the cytochromes P450IA (26,38,46,48). The 4'-hydroxyl moiety is subsequently sulfated or glucuronidated to produce several stable excreted metabolites with 4'-PhIP-sulfate [4'-(2-amino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine)-sulfate] being the predominate metabolite detected in plasma, bile and urine (37,38,48,49). Also detected and identified in urine, plasma and bile are the 4'-PhIP-O-glucuronide [2-amino-4'-(b-1-glucosiduronyloxy)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine], and 4'-hydroxy-PhIP (38,48). Glucuronidation of the N^2- and N^3-positions of the imidazole ring system of the N-hydroxylated PhIP molecule [2-(N-b-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-b]pyridine and 3-(N-b-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-*b*]pyridine, respectively have also been reported (38,45). Analysis of feces has shown primarily 4'-hydroxy-PhIP and PhIP to be present (38,48). These metabolites may be useful in comparing metabolism among species and in predicting susceptibility since they can be easily measured in urine, blood, and breast fluids.

The N^2-PhIP-O-glucuronide and the N^3-PhIP-O-glucuronide, like the N:O-acetylated PhIP, may be meta-stable transportable PhIP metabolites. Meta-stable metabolites may serve to cause damage in tissues where PhIP metabolism does not occur. Indeed, such meta-stable metabolites have been suggested as transportable forms of other N-hydroxylamines which are liberated following hydrolysis in extrahepatic tissues (50, 51). These metabolites may be causal factors for the DNA damage seen in the blood cells of primates and rodents given PhIP and for DNA and protein damage in tissues where PhIP metabolism does not occur (38). Importantly, PhIP's metabolism has primarily been established using liver tissue fractions and male animals. Few data are available on the metabolism of PhIP in breast tissue or on metabolite levels in breast fluids. These data are needed to understand PhIP's mechanism of action in inducing breast tumors and for understanding if breast fluids can be used in molecular epidemiology studies. The data gathered through this project will specifically fill in these data voids such that the role of compounds like PhIP in breast cancer can be better understood and used to predict, on an individual basis, who may be at risk. If such an approach proves feasible, it will help be useful in cancer prevention efforts.

DNA and protein damage

Exposure to PhIP results in DNA, and likely protein, adduct formation. However, little is known about the identity and sequence specificities of nucleic acid and protein adducts, and in which tissues these most easily form. In addition, tissue specificity in

DNA repair is poorly understood. Macromolecular adduction is important since it indicates the active dose of a chemical reaching its target, and is thought to be the initiating event in chemical carcinogenesis. DNA adduct formation with MeIQx has been shown to be quantitatively, but not qualitatively, affected by metabolic capacity (52). PhIP adduct formation may be similarly affected but has not been investigated. N-(deoxyguanosin-8-yl)-3'-monophosphate adducts of IQ, MeIQx and PhIP have been identified and found *in vivo* (39,53-56). Other PhIP adducts also exist and are likewise due to binding at guanines (57). A deoxyguanosin-N^2-yl-PhIP adduct may exist since deoxyguanosin-N^2-yl-MeIQx and MeIQ adducts have been reported.

While most data on the adducts have been derived from studies in the liver, IQ, PhIP and MeIQx have been shown to form adducts in extrahepatic tissues of the rat (12, 31, 56). High levels of PhIP adducts have been found in the large intestine, white blood cells, pancreas, and heart, followed by stomach, small intestine, kidney, and liver (12,56,58). Some mutational sequence specificity has been demonstrated for *Salmonella* DNA with IQ and PhIP and both inducing GC deletions in the standard frameshift sensitive and uvrB-deficient strains TA98 and TA1538 (59). Protein binding has also been suggested for PhIP (38,60) but, to date, has only been unambiguously demonstrated for IQ (61). A major limitation of the data described above is that all have been derived from high-dose studies and no studies have been reported in the breast even though PhIP causes breast tumors. Thus, little can be determined about the toxicity, biochemistry, and macromolecular targets of PhIP in the breast at human dietary doses.

Human tumorigenesis, genotoxicity, and metabolism

Inadequate data exist on the metabolism and pathologies of all the AIA, including PhIP, in humans. Several studies have been conducted which show that increased mutagenic activity and some heterocyclic amines can be detected in the urine of fried-meat eaters and men on normal diets, although metabolite recoveries tend to be poor (1, 62-64). Purified human cytochromes P450 and human tissue fractions have been shown to oxidize the AIAs to mutagenic intermediates *in vitro* (65-69). Specifically, liver fractions are known to form the N-hydroxy-PhIP metabolite (70). Further, purified acetyltransferases from human tissues have been used to show that N-hydroxy-PhIP is probably acetylated by the polymorphic arylamine acetyltransferase (71). The paucity of human data can be partially attributed to technical difficulties in measuring metabolism at the low heterocyclic amine concentrations that people are naturally exposed to, and to the difficulty in obtaining material from human subjects. Such difficulty is often methodological in nature. A major goal of the work proposed here will be the development and validation of methods which will allow detection of molecular effects in easily accessible human tissues, such as breast fluids and blood. Development and validation of such methods are important for comparing animal and human metabolism, assessing inter-individual differences in metabolism and for eventual use in identifying high risk individuals, since individual differences in metabolism represents a potentially important determinant in risk associated with carcinogen exposure (72).

PURPOSE AND SCOPE OF THE RESEARCH

The scope of this proposal is to determine if the dietary breast carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) causes macromolecular damage in the breast, and the mechanism by which this damage occurs at human levels of

exposure. Our purpose was to define the molecular events leading to the development of PhIP-induced breast tumors, and to assess the likelihood that PhIP exposure at human dietary levels present a human breast cancer risk. A crucial step in risk determination is the estimation of the dose of a reactive carcinogen reaching the critical molecular target. DNA adducts are particularly relevant for this purpose since the adduct, if not repaired, can be considered the initial step in the multistage process of cancer. Protein adducts may likewise be useful since they are indicators of the active carcinogen dose in the tissues. Our goals are to understand the effects of chemical dose (exposure) on adduct formation and metabolism, the types of adducts formed, how adducts are repaired, and the ability of the breast to metabolize PhIP at exposure levels expected to occur via the human diet. This low-dose work is possible by use of AMS, a highly sensitive and novel technique for tracing ^{14}C-labeled xenobiotics with sensitivity in the zeptomole (10^{-21} moles) range. The data collected through this project will help determine if exogenous factors present in the diet can be linked to breast cancer and how best to extrapolate breast cancer risk from standard high-dose tumor assays. Further, this work will lead to a better understanding of the utility of using adducts or metabolites for identifying women at risk for cancer, either because of exposure to high levels of exogenous compounds or due to metabolism genotype. Finally, the data gathered through this work will be used to develop a sensitive assay for assessing PhIP metabolism, exposure and, potentially, risk in humans. If successful, this work will lead in out years to directly studying the molecular epidemiology of PhIP in human breast samples and to defining the role of compounds like PhIP in the etiology of breast cancer.

BODY

The progress made towards each of the specific aims of the research project completed in the period July 1994 to September 30, 1998 are described as follows:

1. Ultra-low level pharmacokinetics by accelerator mass spectrometry

The first specific aim is to determine the pharmacokinetics of PhIP in rodents at low doses. We have studied the pharmacokinetics of PhIP following acute oral administrations of PhIP and in chronic feeding experiments. We have included both male and female F344 rats to provide useful comparisons between gender and to credibly assess our results versus published data performed with higher doses.

Pharmacokinetics of PhIP following acute oral administration

In order to examine the amount of PhIP reaching the breast tissue in rodents as a function of dose, female F344 rats were acutely dosed by gavage with [^{14}C]PhIP in the dose range 5 ng/kg to 100 mg/kg [36 animals total, 3 animals/dose group], a dose range that incorporates environmentally relevant and rodent bioassay levels. The [^{14}C]PhIP utilized has a specific activity of 10 mCi/mmol, with doses above 10 µg PhIP/kg serially diluted in unlabeled PhIP. Animals were sacrificed 6 hours after dosing, a time point chosen to reflect the initial peak of tissue uptake. Liver, breast and colon, which included target and non-target organs for PhIP induced carcinogenicity, were removed and analyzed by AMS (figure 1).

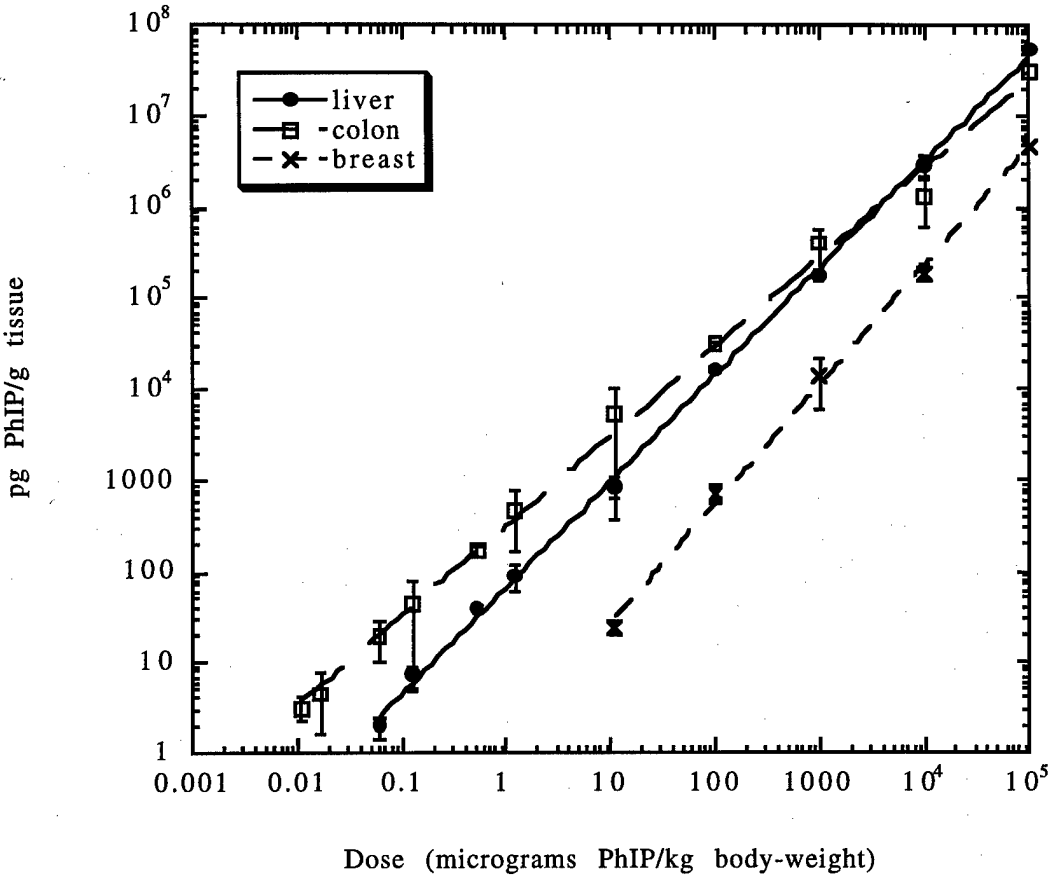

Figure 1. Dose-response for [^{14}C]PhIP in female F344 rats. Data from liver, colon and breast tissue following a single acute dose are shown. Lines illustrate the power fit and the data points are the means ± SD of three replicate animals.

This acute PhIP dosing study demonstrated that PhIP is distributed to both the colon and breast tissue, the target organs for PhIP-induced carcinogenicity in female rats. Furthermore, the levels of PhIP in the breast and colon tissues increased as a linear function of dose in the dose range 5 ng/kg to 100 mg/kg. The mean breast tissue levels were 4.63 x 10^6 pg PhIP/g tissue at 100 mg/kg dose and 17 pg PhIP/g tissue at 10 µg/kg dose. Below 10 µg/kg binding was not detectable in the breast tissue, which may partially be due to the very high levels of carbon in this tissue [67.7% carbon] compared to the liver [29.7% carbon] and colon [11.8% carbon], which would effectively dilute any ^{14}C signal. Initially, combustion of breast tissue samples prior to AMS measurement was problematic, as combustion tubes frequently exploded. This was considered to be due to the very high carbon levels which resulted in an excessive amount of CO_2 being produced during the combustion process. Further analyses were completed with reduced amounts of breast tissue compared to liver and colon samples.

Exposure to PhIP in the diet causes breast tumors in female rats, but not in males (13). Therefore, in order to investigate if differences in PhIP bioavailability in the breast tissue may account for these sex differences in carcinogenicity, the acute PhIP dosing study was repeated in male F344 rats. The results of AMS analysis of liver, colon and breast tissue of male rats are presented in figure 2.

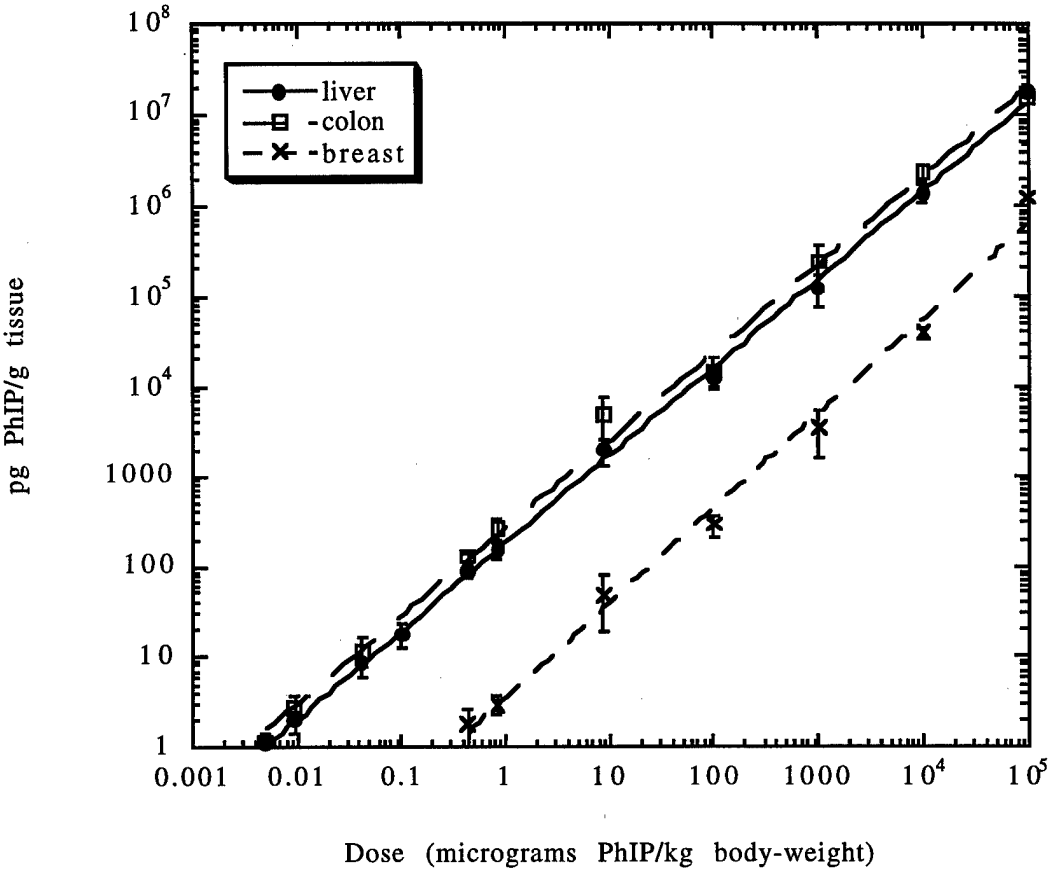

Figure 2. Dose-response for [^{14}C]PhIP in male F344 rats. Data from liver, colon and breast tissue following a single acute dose are shown. Lines illustrate the power fit and the data points are the means ± SD of three replicate animals.

The concentration of PhIP in the liver, colon and breast tissue samples increased linearly with administered dose. The highest levels of PhIP in the dose range 5 ng/kg to 100 mg/kg were observed in the liver and colon tissue. Fundamentally, the breast tissue of male rats contained measurable levels of PhIP. The mean breast tissue levels were 1.23×10^6 pg PhIP/g tissue at 100 mg/kg dose and 48.20 pg PhIP/g tissue at 10 µg/kg dose. Below 500 ng/kg ^{14}C-PhIP was not detectable in the breast tissue. However, due to the higher specific activity of [^{14}C]PhIP used in this study, we could detect [^{14}C]PhIP at much lower levels in the breast tissue than in the previous female rat study.

It can be seen that for both male and female rats there is a clear dose-response for levels of [^{14}C]PhIP in the breast tissue following an acute exposure to PhIP.

Interestingly, the slope of the dose-response curve is greater for the female rats than the male rats (Figure 3). For example, at the higher PhIP doses it appears that bioavailability of PhIP to female breast tissue is greater than to male breast tissue. Therefore, although at high doses (mg/kg body-weight) there appear to be significant sex differences in bioavailability, at the lower doses the tissue bioavailability alone dose not appear to clearly indicate that female rats would be more likely to develop breast tumors. Furthermore, the dose-response curves for PhIP levels in colon tissue in females and males were very similar (plot not shown). Both male and female rats develop colon tumors following chronic exposure to PhIP, although males have been shown to have a higher incidence than females (13). Therefore, the results indicate that tissue bioavailabilty alone does not account for these sex differences in PhIP induced carcinogenicity in the colon. In the liver, it appears that female rats have consistently lower levels of PhIP (plot not shown), although the liver is not a target organ for PhIP carcinogenicity in either sex.

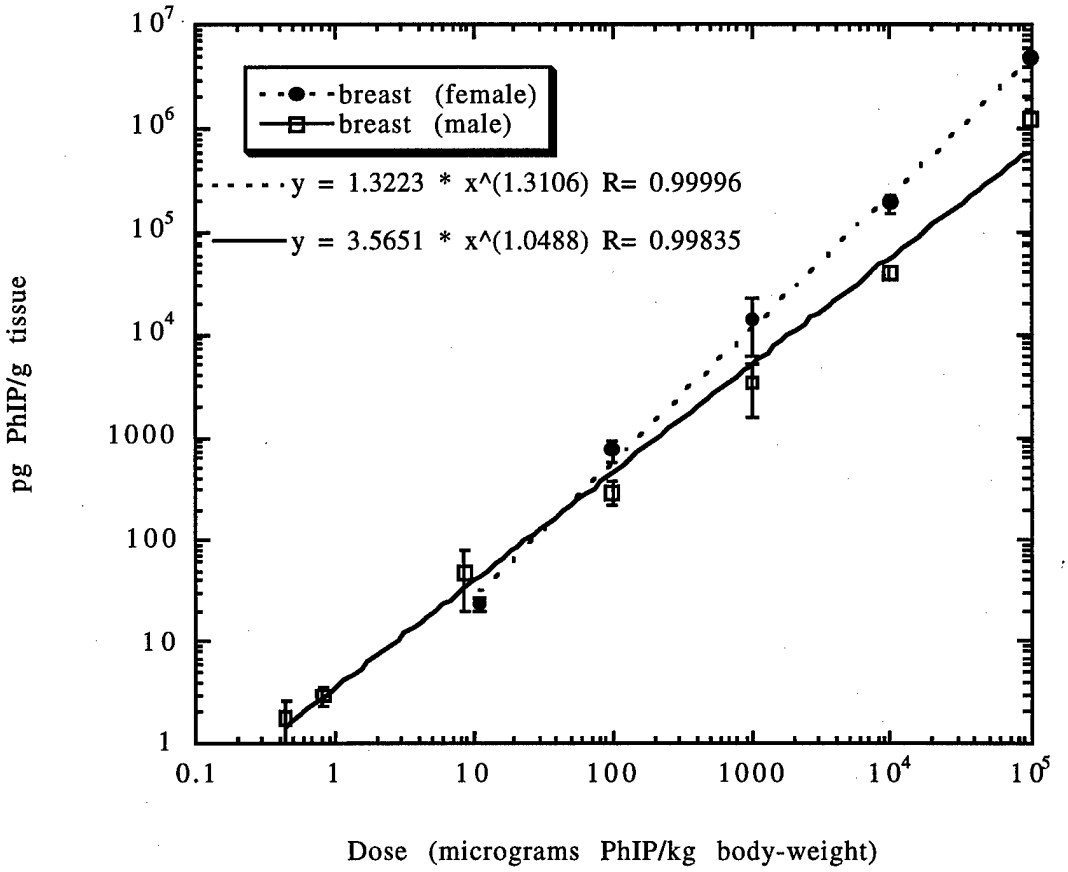

Figure 3. Summary of dose-response for [^{14}C]PhIP in the breast tissue of male and female F344 rats following a single, acute dose. Data points are the means ± SD of three replicate animals. Lines and equations represent the power fits of the data.

In conclusion, the acute PhIP dosing studies have demonstrated that PhIP is distributed to breast tissue in both male and female rats. Furthermore, the levels of

PhIP in the breast tissues increase as a linear function of dose. Although the slopes of the dose-response curves are slightly different between the sexes, there appear to be no clear differences in the data that would suggest why female rats are at greater risk of breast cancer than male rats. Furthermore, there appear to be no differences in the amounts of PhIP distributed to the colon between the male and female rats, demonstrating that levels of PhIP bioavailability also do not correlate with carcinogenicity in this organ. This work was presented at the American Association for Cancer Research 1998.

Pharmacokinetics of PhIP in chronic feeding experiments

The goal of these studies was to determine the steady-state levels of PhIP available to the tissues, to determine when adduct levels reach equilibrium in the tissues, and to study the rate of clearance of these compounds. Male F344 rats (200 g) were placed on a powdered rodent chow diet modified with [^{14}C]PhIP (10 ng/kg/day to 1 mg/kg/day) for up to 42 days. This dose range spans exposure levels from human equivalent levels through levels shown to induce tumors in this rat strain. Rats were sacrificed at days 0, 1, 3, 7, 14, 21, 35 and 42 to determine tissue distribution and DNA adduct kinetics. The animals were then switched back to a standard rodent chow not containing PhIP to examine clearance kinetics and were sacrificed after an additional 1, 3, 7, 17, and 35 days. At the time of sacrifice, blood and tissues were collected and frozen at -20°C until analyzed for radiocarbon content by AMS or extracted for DNA.

Analysis of tissues from rats administered a low dose of PhIP shows that the highest levels of PhIP are found in the intestine (Figure 4). Furthermore, we have shown accumulation of PhIP in some tissues (liver, kidney) increases through 2 weeks and then maintain a steady state while others (blood, spleen) continue to increase through the entire 6 weeks (Figure 5). At a dose of 500 ng/kg bw/day, steady state levels in the liver average approx. 450 pg PhIP/g liver while blood levels reach a maximum level of approx. 50 pg PhIP/g blood at 42 days. After animals were taken off the PhIP-modified diet, adduct clearance varied in the different tissues with rapid clearance from the liver and spleen and slower clearance from kidney and blood. Notably, all tissues measured remain above background levels 4 weeks after removal of the PhIP-modified diet. At 28 days after removal of the PhIP-modified chow, the liver retains 26 pg PhIP/g liver while the spleen (160 pg/g) and kidneys (250 pg/g) maintain a higher steady state level of PhIP or PhIP-derived materials. DNA adducts from these tissues show a continual increase in liver and colon adduct levels through 35 days. A subset of the animals in these experiments were also housed in metabolism cages to assess the absorption and clearance of the administered dose into the urine and feces. For the doses analyzed, approximately 5%/day of the dose is eliminated through the urine while approx. 50%/day is eliminated in the feces. In comparison, acute studies have shown that 78% of the dose is excreted in the feces (73). These results were presented at the American Association for Cancer Research, 1997.

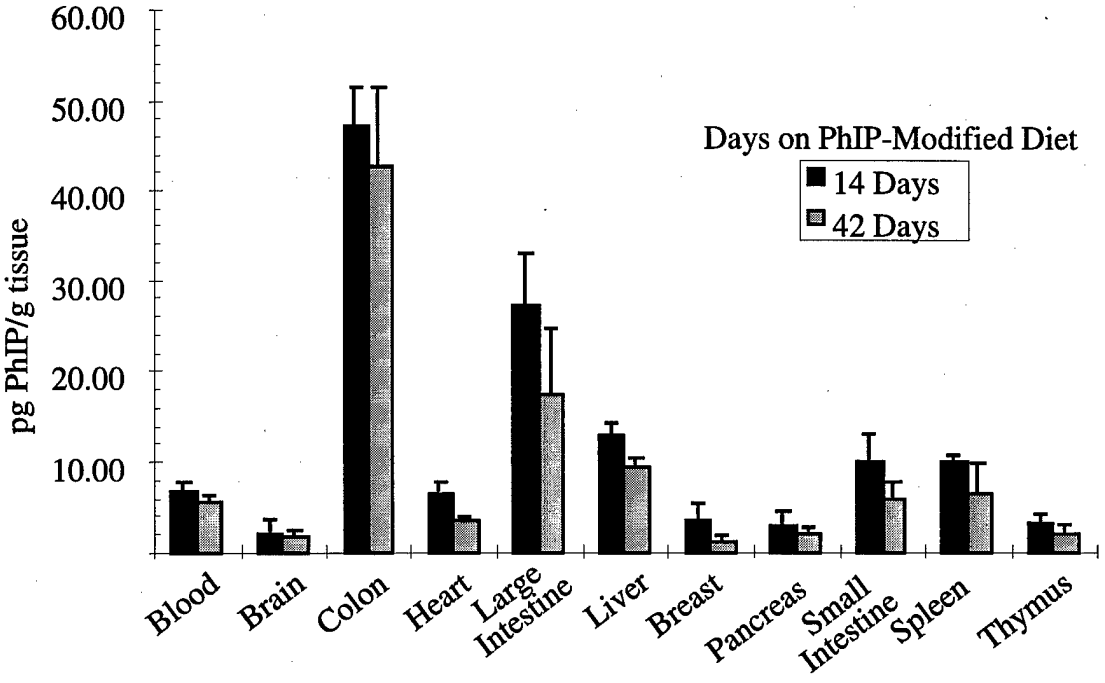

Figure 4 : Levels of [^{14}C]PhIP and PhIP derived products in F344 rat tissues consuming 20 ng PhIP/kg body weight/day for 14 and 42 days.

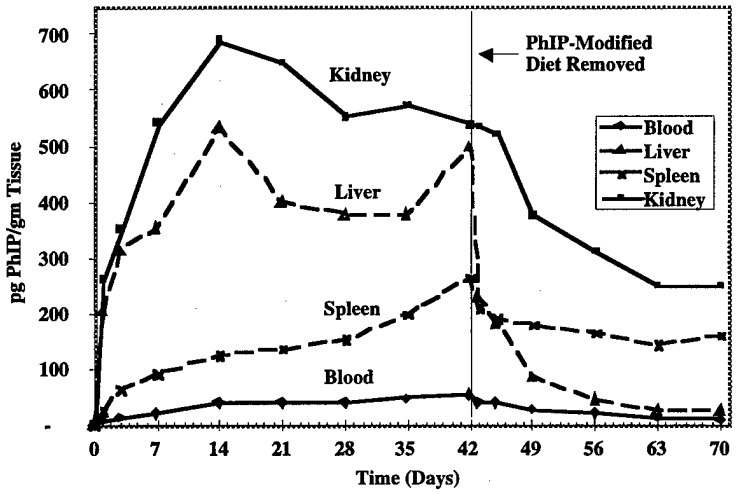

Figure 5 : Accumulation and clearance of [^{14}C]PhIP and PhIP derived products in F344 rat tissues consuming 500 ng PhIP/kg body weight/day for 42 days. At day 42, normal rodent chow was returned.

2. Breast metabolism of PhIP

Towards specific aim 2 we have conducted a study to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups. Additionally we are developing HPLC/AMS separation protocols for determination of metabolite levels in the milk from these animals. Lactating female F344 rats with suckling pups were gavaged with doses ranging from 50-1000 ng/kg [^{14}C]PhIP. The excretion of the [^{14}C]PhIP in the milk and distribution of [^{14}C]PhIP into the breast tissue, liver and blood of the dam as well as in the stomach contents, liver and urine of their suckling pups were measured using AMS (Figure 6).

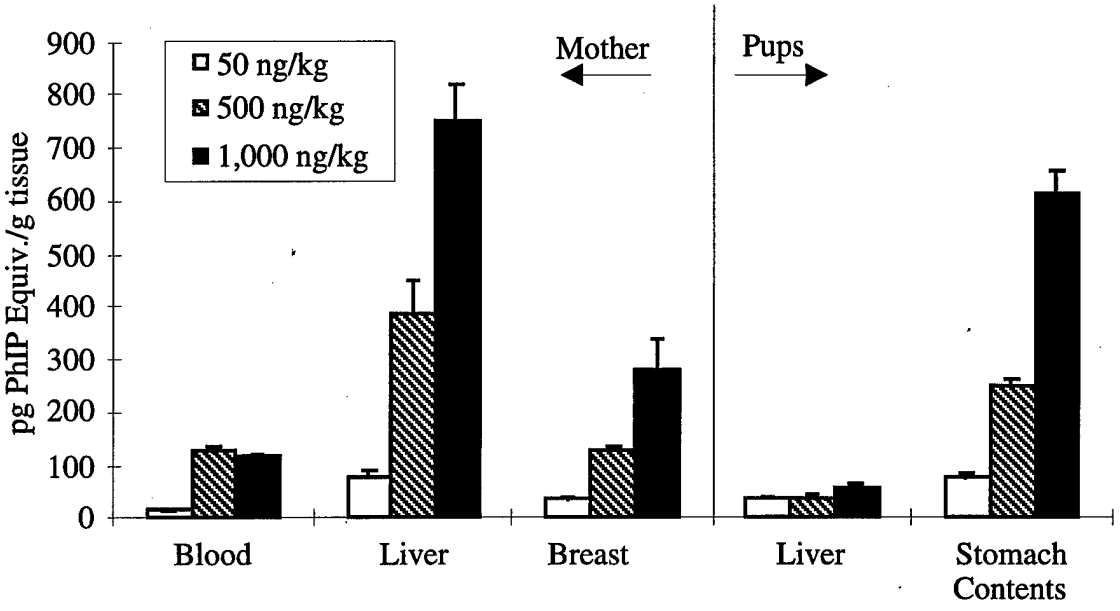

Figure 6. Distribution of [^{14}C]PhIP in lactating female rats and their suckling pups. Data points for Dams are the means ± SD of three replicate animals. Data points for the pups are from three individual pools of pup litters.

[^{14}C]PhIP-derived radioactivity increased in a dose dependent manner in both the milk and stomach contents of the pups as well as in the other tissues measured. Highest levels of PhIP related radioactivity were found in the liver with levels of approximately 750 pg PhIP equivalents/g liver tissue for the 1000 ng/kg dose. Although significantly lower, the breast tissue contained high levels of PhIP derived material with levels of approx. 300 pg/g breast tissue at the 1000 ng/kg dose. Separation of the individual PhIP metabolites by HPLC and analysis of the radiocarbon content of the collected fractions by AMS indicate 2 major metabolites in the milk (4'-hydroxy PhIP and an unidentified peak). Lactating female rats also were dosed with 500 mg/kg chlorophyllin in conjunction with a 500 ng/kg [^{14}C]PhIP dose. The chlorophyllin treatment caused increased levels of [^{14}C]PhIP in the milk and stomach contents of the pup while decreasing levels in all other tissues measured (Figure 7). The results from these studies suggest that at dietary levels of PhIP, PhIP and/or PhIP metabolites are excreted into the breast milk and absorbed by the newborn. The findings raise the possibility that there is a carcinogenic risk to the newborn by exposure to low levels of PhIP via the breast milk. The addition of

chlorophyllin to the dosing regimen demonstrates that other components in the diet may modulate the excretion of [^{14}C]PhIP-derived radioactivity into the breast milk and alter the uptake into tissues of newborns. The effects of addition of chlorophyllin has implications for chemoprevention strategies. A manuscript describing these studies has been published (Mauthe *et al.*, 1998).

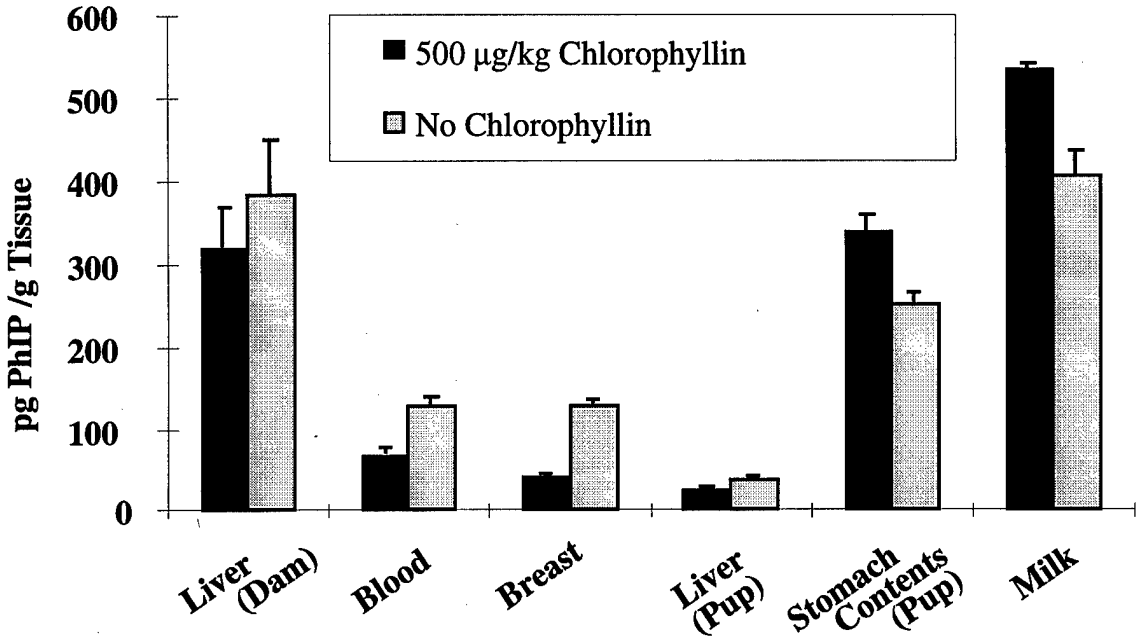

Figure 7. Effect of 500 mg/kg chlorophyllin cotreatment with 500 ng/kg [^{14}C]PhIP treatment. Data points are the means ± SD of three replicate animals.

3. Determination of DNA and protein adducts of PhIP in the breast

The third objective of these studies is to determine if adducts are formed in rodents, particularly in the breast at low dose and to characterize the adducts. To date, only 1 DNA-adduct has been structurally identified, although several more may form *in vivo*. Therefore, there is a requirement for chemically synthesized adducts which will be employed as standards.

Synthetic DNA adducts have been produced by the reaction of N-hydroxy PhIP and N-acetoxy PhIP with calf thymus DNA and deoxyguanosine and the spectra of adducts produced analyzed by absorption and fluorescence spectroscopy, as well as ^{32}P-postlabeling (Marsch *et al.*, 1995). It was established that DNA modification by N-hydroxy PhIP was 20-50 times less efficient than by the further esterified N-acetoxy PhIP, although the spectra of adducts produced by both appeared identical. Primarily dG-C8-PhIP adduct was formed by both species, with small yields of 3 other adduct species. Adduction in MOPS or phosphate buffers (pH 6.5-7.0) compared to citrate buffer (pH 5.0) resulted in greater yields of the 3 minor adducts compared to the dG-C8-PhIP adduct. Furthermore, these adducts were consistent with the DNA adducts observed in PhIP dosed animals. A fourth adduct was detected in N-hydroxy PhIP modified DNA, which was not seen in N-acetoxy PhIP modified DNA or *in vivo*.

Further work has been undertaken to fully characterize the minor DNA adducts present in N-acetoxy PhIP modified DNA. In order to do this, bases, deoxyribonucleosides and deoxyribonucleotides have been adducted and analyzed by mass spectrometry. Data to date, suggests PhIP forms one major adduct which we have confirmed as the dG-C8 guanine adduct, and 4 minor adducts seperable by HPLC. Each of the minor adducts has an m/z of 507 but unique fragmentation pattens by ES-MS analysis. Initial data suggested one is a ring opened adduct. This work was presented at the American Association for Cancer Research, 1996.

In preparation for NMR studies, adduction experiments were conducted utilizing an 11 base pair double-stranded oligonucleotide containing a 5'-GGA-3' 'hot-spot' for PhIP modification. HPLC analysis of the digested adducted oligonucleotide peaks suggested that mainly deoxyguanosine was modified by N-acetoxy-PhIP and that in many cases the adduct was the dG-C8 PhIP adduct. Other adduct peaks appeared to be very unstable and yielded inconclusive digestion results.

We have developed an improved HPLC-based postlabeling assay for measuring the individual PhIP-DNA adducts in animal models. With HPLC Inline Precolumn Postlabeling (HIPP) and a sensitivity of approximately 1 adduct/10^9 bases, we are able to measure PhIP-DNA adducts in animal models, cell culture systems, and tissue slices. Postlabeled samples are loaded onto a C_{18} precolumn and adducted bases are retained while excess radioactivity and the unmodified bases are eluted through a UV detector to waste through a switching valve. The use of this inline precolumn enrichment allows entire postlabeled samples to be analyzed without prior purification of labeled adduct and also allows determination of the exact amount of sample loaded onto the column. The adducted samples are then eluted onto an analytical reversed phase column to separate the individual PhIP-DNA adducts (Figure 8).

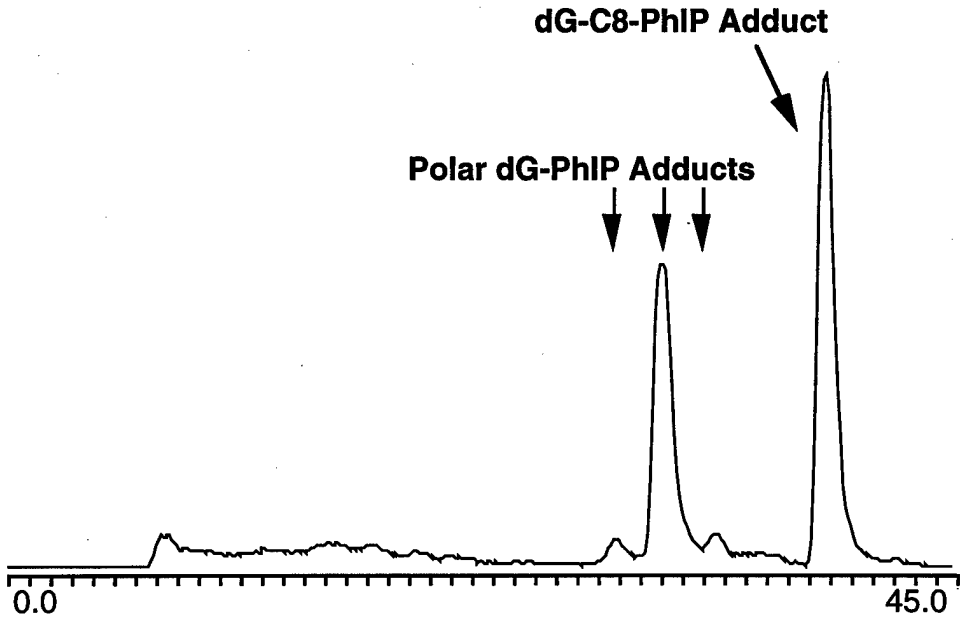

Figure 8. Typical ^{32}P-postlabeling profile for PhIP modified DNA using newly developed HPLC Inline Precolumn Postlabeling (HIPP) methods.

PhIP-DNA samples show 2 major peaks and up to three additional minor adduct peaks when labeled under ATP-limiting conditions. The method has a sample to sample standard error of 10 percent at adduct levels of 1 adduct/10^7 bases and shows a linear

relationship between signal and adduction levels down to approximately 1 adduct per 10^9 bases. Individual DNA samples (1 to 25 μg) can be analyzed by HPLC in less than 1 hour allowing high throughput of postlabeled samples. Extensions of this technique designed to measure overall adduct levels rather than separate specific PhIP-DNA adducts allows for 15 minute analysis times (rapid-HIPP). These short analysis times allow for more replicates to be measured yielding higher accuracy and precision in the measurements. For example, DNA adduct levels in a study where rat colon slices were exposed to N-OH-PhIP were determined with less than 10% errors using the rapid-HIPP method (Figure 9). These extremely accurate postlabeling values are typically very difficult to achieve using standard postlabeling assays and allows for a much wider variety of studies to be performed. In addition to the high resolution provided by HPLC separation of the PhIP-DNA adducts, this method can be adjusted for analysis of other DNA adducts and is readily automated for high throughput and decreased handling of ^{32}P. A manuscript describing this technique has been published (Mauthe *et al.*, 1996).

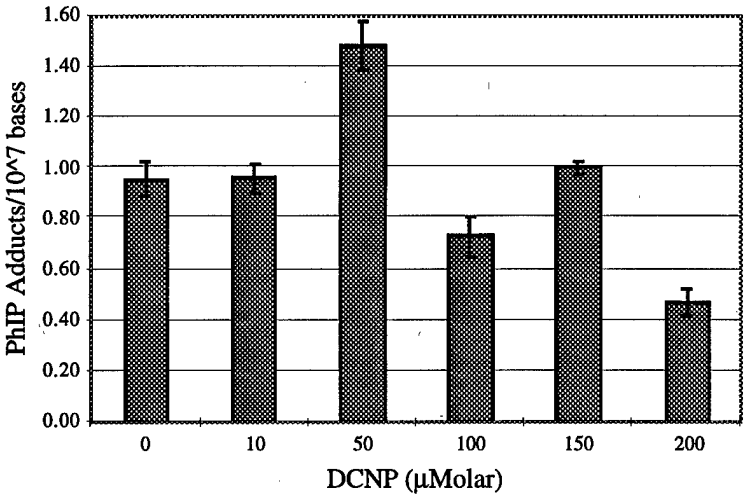

Figure 9. ^{32}P-postlabeling results demonstrating high accuracy of rapid-HIPP assay. Errors average approx. 6%. In this case, colon slices were incubated with N-OH PhIP and increasing concentrations of DCNP, a phase II conjugation inhibitor (Malfatti et al., (1996) Cancer Research vol 56, 2550-5).

4. Dose-response relationships

Objective 4 is concerned with the understanding of the effects of the dose of PhIP on DNA adduct formation in breast and non-breast tissues, hence establishing data to be employed for the extrapolation to breast cancer risk in humans at low-dose exposures.

As part of these studies, the dosimetry of PhIP on PhIP binding to the DNA in the liver, colon and breast tissue is being determined in an acute PhIP dosing study [for details of the dosing regimen refer to objective 1]. DNA from liver, colon and breast tissue samples in this study were extracted and the covalent [^{14}C]PhIP binding measured by AMS. Initial difficulties have necessitated the investigation of additional purification steps in the DNA extraction procedure. Furthermore, liver DNA initially provided low ^{14}C signal requiring adapation of the sample preparation methods so

that undiluted DNA could be measured [which requires approximately 500 μg DNA]. These new methods increase the sensitivity and accuracy of the measurement and allow a maximum sensitivity of approximately 1 adduct/10^{11} nucleotides (with 10 mCi/mmol [^{14}C]PhIP). Unfortunately, this methodology could not be utilized with breast DNA due to the relatively low yields of DNA [0.1-0.3 mg DNA/g tissue]. Analysis of the breast DNA with a reduced amount of tributyrin carrier, but sufficient for graphitization, was used. The results of the breast, colon, and liver DNA analyses are shown in figure 10.

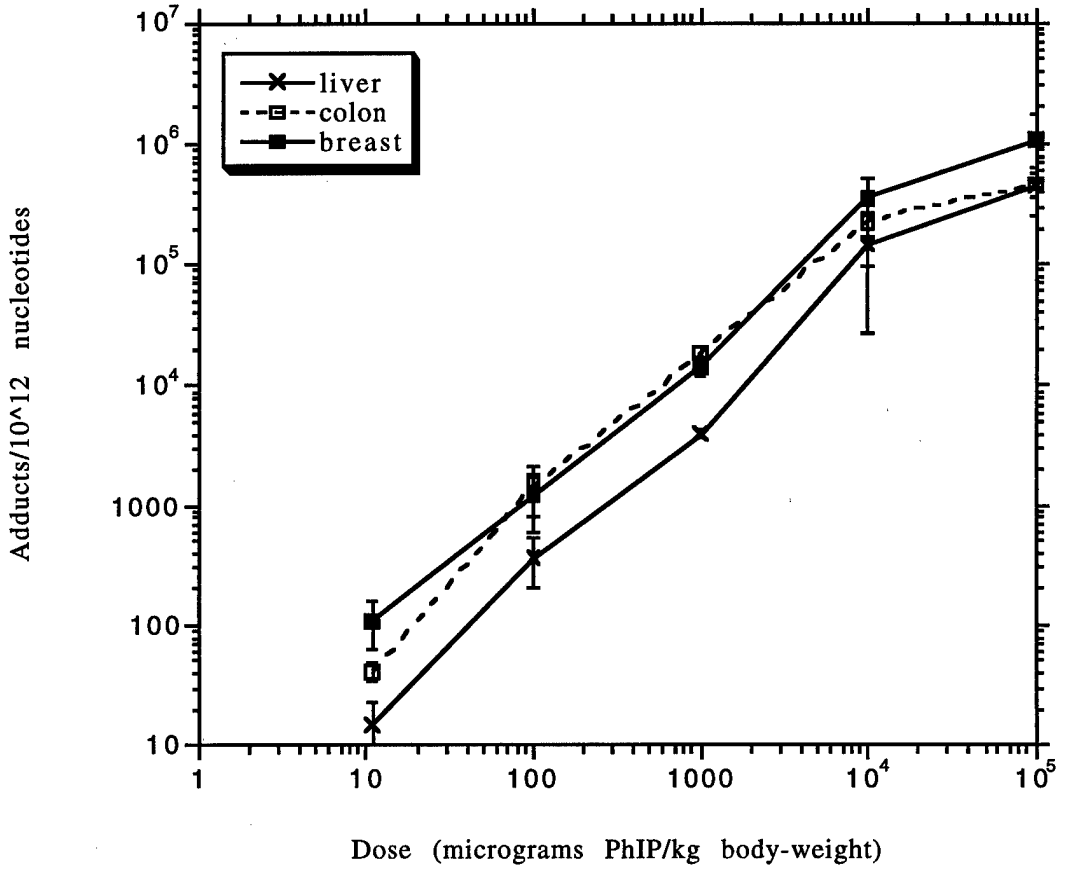

Figure 10. Dose-response curves for DNA adduct formation by [^{14}C]PhIP in female F344 rat breast, liver and colon following a single acute dose. The power fit lines are shown. Data are means ± SD of three replicate animals.

Analogous to the tissue binding data, the dose-response curves for DNA adduct formation in the liver, colon and breast were dose-dependent over the measurable range with a mean peak adduct level at 10 μg/kg dose of 15.2, 41.5 and 139.6 adducts/10^{12} nucleotides in the liver, colon and breast, respectively. DNA adducts were not detectable below 10 μg/kg using this acute dosing regimen.

In order to look at sex differences in PhIP distribution and macromolecular binding, the acute PhIP dosing study was repeated in male F344 rats (see specific aim 1 for experimental details) and the DNA extracted from the liver, colon and breast tissue. The PhIP-DNA adduct levels in these tissues in male rats is shown in figure 11:

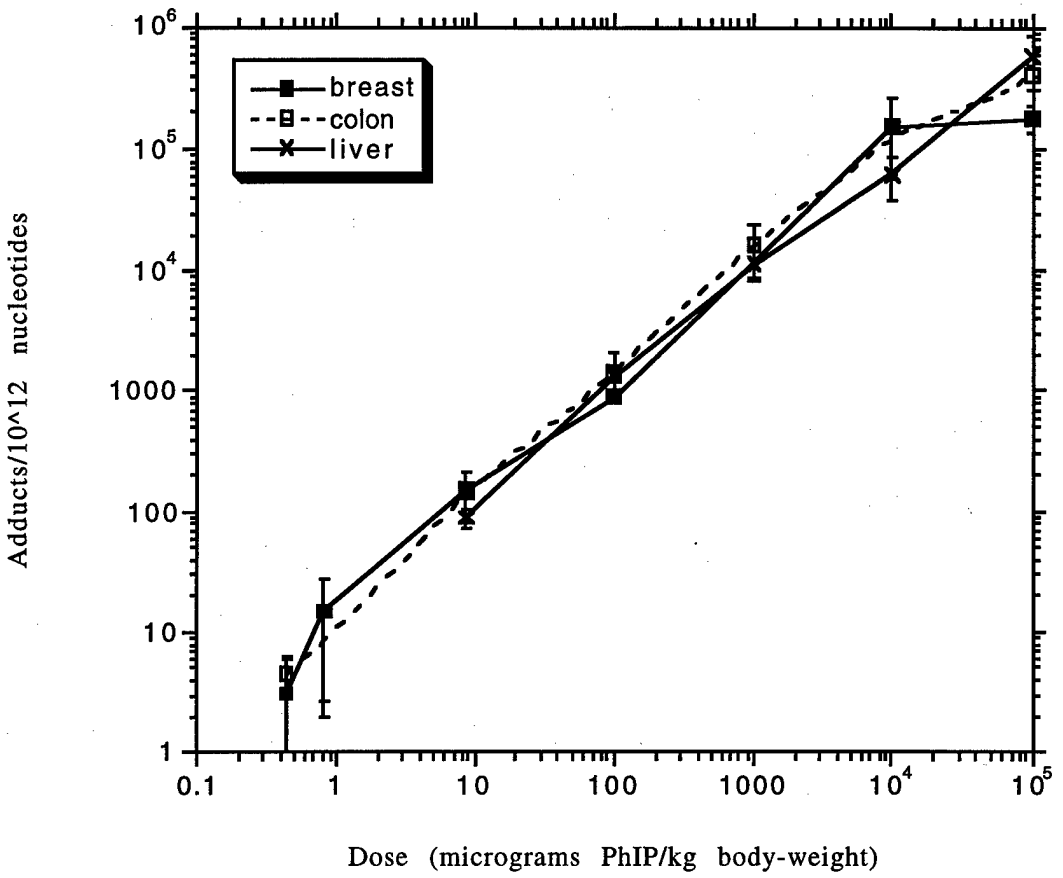

Figure 11. Dose-response curves for DNA adduct formation by [^{14}C]PhIP in male F344 rat liver, colon and breast following a single acute dose. The power fit lines are shown. Data are means ± SD of three replicate animals.

In the male rats, the dose-response for PhIP-DNA adduct formation in the colon, liver and breast were very similar up to a dose of 10 mg/kg. At a dose of 10 μg/kg, the mean adduct levels in the liver, colon and breast tissue were 67.2, 145.1 and 143.1 adducts/10^{12} nucleotides respectively. This would imply that DNA adduct levels do not correlate with PhIP induced carcinogenicity in the male rat using this dosing regimen.

In order to investigate if there are sex differences in PhIP-DNA adduct levels in the breast tissue between the male and female rats that may account for the differences in PhIP's carcinogenicity, the dose-responses for PhIP-DNA adduct formation in this

organ were compared (figure 12). At doses of 10-100 mg/kg in the male rats the dose-response plateaued.

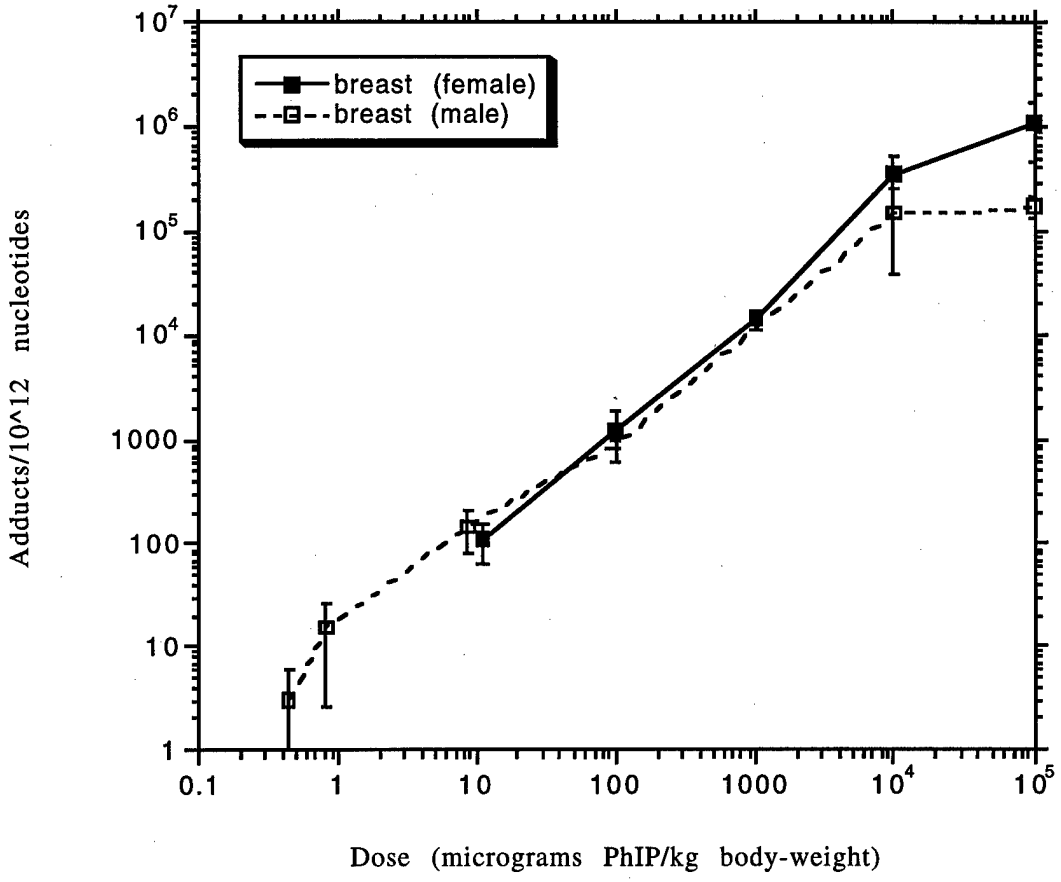

Figure 12. Dose-response curves for DNA adduct formation by [^{14}C]PhIP in male and female F344 rat breast tissue following a single acute dose. The power fit lines and equations are shown. Data are means ± SD of three replicate animals.

From the comparison of the DNA adduct formation in the male and female rat breast tissue, it can be seen that DNA adduct formation is similar up to a dose of 10 mg/kg. At a dose of 100 mg/kg DNA adduct levels are significantly greater in the female breast tissue, whereas adduct levels reached a plateau in the male breast tissue, indicating possible saturation of enzymes involved in metabolic activation of PhIP.

In conclusion, PhIP formed DNA adducts in the liver, colon and breast tissue in both male and female rats following acute oral exposure. In the female rats, the breast tissue had the highest levels of DNA binding, followed by the colon and then liver. Levels of DNA binding in these tissues were all dose-dependent in the dose range measured. In the male rats adducts were also detected in the liver, colon and breast tissue. Unlike the female rats, adduct levels detected in these organs were similar in males. Comparing the male and female breast adduct levels, the female breast tissue had higher levels of binding than the males at the 100 mg/kg dose, but were similar at lower doses. This suggests that at high dose the female rats may be at greater risk

from PhIP-induced breast tumors. This work was presented at the American Association for Cancer Research 1998.

Protein adduct formation by PhIP

In order determine if PhIP binds to protein in rats dosed with PhIP and if protein adducts could be used as biomarkers of exposure or surrogates of DNA damage at low dose, we extracted albumin and hemoglobin from the blood of rats dosed with 10 ng/kg-10 μg/kg [^{14}C]PhIP and measured the samples by AMS (figures 13 and 14). The data demonstrated that protein adduct formation does occur at low dose and is dose-dependent. Furthermore, albumin adduct levels are higher than hemoglobin adduct levels. These studies demonstrate that albumin adduct formation by PhIP might be useful as a marker of exposure and PhIP bioactivation for use in molecular epidemiology studies.

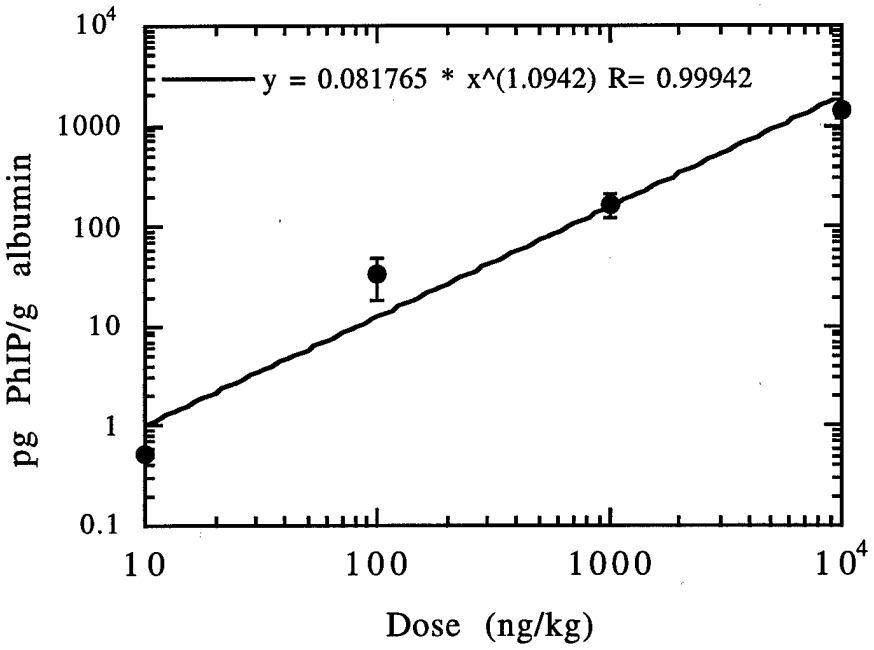

Figure 13. Dose-response curves for albumin adduct formation by [^{14}C]PhIP in rats following a single acute dose. The power fit line and equation are shown. Data are means ± SD of three replicate animals.

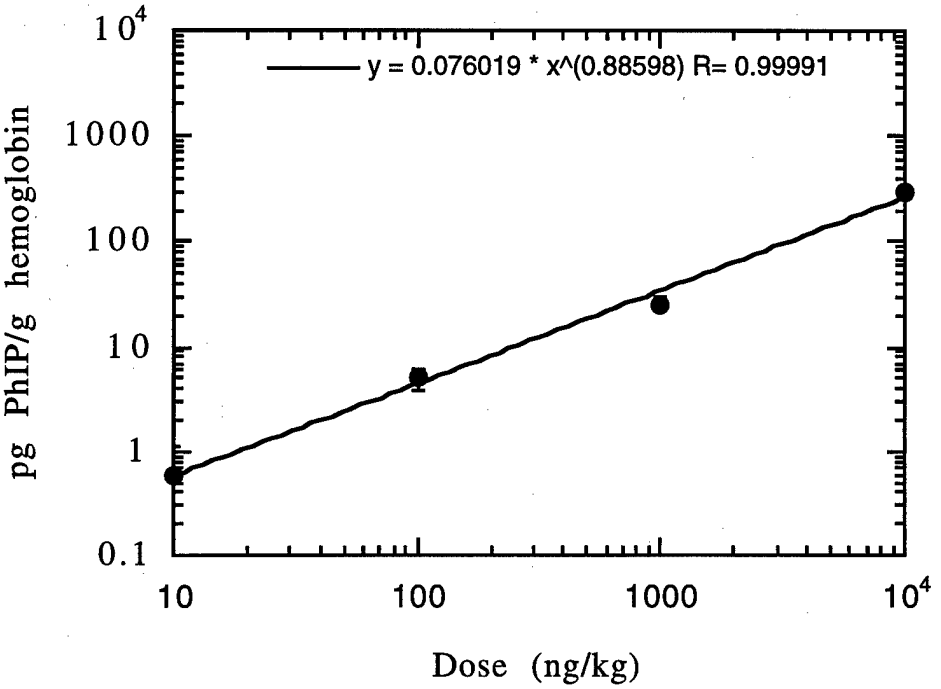

Figure 14: Dose-response curves for hemoglobin adduct formation by [^{14}C]PhIP in rats following a single acute dose. The power fit line and equation are shown. Data are means ± SD of three replicate animals.

5. Development of an AMS isotope-labeled immunoassay

The purpose of specific aim 5 was to produce antibodies against PhIP modified DNA. The antibodies can then be utilized in a selective and sensitive immunoassay to detect and quantify PhIP-DNA adducts in various biological samples from laboratory animal and human studies. The immunoassay could potentially have applications in a wide range of molecular epidemiology studies to investigate the link between breast cancer and PhIP exposure. For example, in the validation of PhIP-DNA adducts as biomarkers in assessing exposure to PhIP and in determining susceptibility to breast cancer. In addition, it may also be useful for assessing the effectiveness of cancer chemopreventive agents.

Towards meeting the goals of specific aim 5 we have conducted several studies to determine the total number of adducts formed and the optimal conditions for making PhIP DNA adducts with intact DNA. Our goal was to identify which adduct is present in highest yield and to have conditions to make this adduct for use in immunization of rabbits for production of anti-sera. N-hydroxy-PhIP was produced by controlled reduction of nitro-PhIP using palladium/carbon as a catalyst. The resultant N-hydroxy-PhIP was N:O-acetylated by addition of acetic anhydride. The product, N-acetoxy-PhIP, was then added dropwise to solutions of calf thymus DNA. The adducted DNA was solvent extracted, ethanol precipitated, redissolved in buffer, extensively

dialyzed and finally studied using a combination of absorption and fluorescence spectroscopic methods. From these studies 5 DNA adducts were detected. The primary adduct had an absorption max at 395 nm and fluorescence absorption and emission maximums at 365 and 395 nm, respectively. This adduct was tentatively determined to be PhIP-dG-C8. Silver and acrylamide quenching studies showed that this adduct exists in both groove bound and intercalated conformers. The other adducts were not identified in this work. Digestion of this DNA and analysis by ^{32}P-postlabeling also suggested 5 adduct peaks.

We synthesized 15mg of highly adducted PhIP-DNA [approximately 1 adduct/100 nucleotides] and sent it to Miriam Poirier at NIH for antibody production. Analyses of the adduct distribution of the adducted sample was performed by ^{32}P-postlabeling using both TLC and HPLC separation. The HPLC-based postlabeling method employed has been developed in this laboratory and enables sensitive measurements to be made in less than an hour. The spectrum of adducts observed when the adducted sample was analyzed was similar to those previously reported by Marsch *et al.* (1995) for PhIP modified DNA. Three main adduct peaks were detected, the largest one having a retention time that corresponded to the dG-C8 PhIP adduct.

Polyclonal anti-PhIP-DNA antibodies have been successfully produced in Miriam Poirier's laboratory by immunization of rabbits with the PhIP-DNA. These antibodies have been used in a fluoroimmunoassay to detect and quantify the *in vitro* highly modified PhIP-DNA used in antibody production and to assess antibody cross-reactivity. In this assay, 96 well micro titer plates are coated with highly modified PhIP-DNA. Plates are then coated with a blocking agent to reduce non-specific binding of the antibodies to the plates. Serial dilutions of PhIP-DNA in carrier calf thymus DNA are made to achieve standards containing a range of amounts of PhIP-DNA. Antibody is added to these standards (or unknowns) and allowed to equilibrate for 30 minutes. During this time, the antibodies bind to the PhIP-DNA adducts. The mixture is then added in triplicate to the coated titer plate wells and incubated for a further 1.5 hours, in which time any free antibody will bind to the wells in the plate. The wells are then washed to remove unbound material and a secondary biotinylated anti-rabbit antibody added, incubated and washed. A streptavidin labeled enzyme is then added, which binds to the biotin. Finally, a substrate is added, which is converted to a fluorescent product by the enzyme. The fluorescent product is detected using a plate reader. A standard curve is produced using the fluorescence data of the serially diluted standards, which can be used to quantify adduct levels in unknown samples.

The sensitivity of this assay using the highly adducted PhIP-DNA is 33 adducts/10^9 nucleotides, using 20 μg of DNA per sample. Using this assay, the cross-reactivity of the antibodies has been determined with unmodified calf thymus DNA and PhIP instead of PhIP-DNA in the initial incubations. In both cases, no appreciable binding of the antibodies to these compounds was seen, indicating that the polyclonal antibodies produced are specific to PhIP-DNA adducts.

Methods of Procedure used in these studies: Development of tritium AMS

Tritium-Accelerator Mass Spectrometry (^{3}H AMS) has been developed in order to measure the ^{3}H content of mg-sized biological research samples. LLNL has already successfully applied ^{14}C AMS to a variety of problems in the area of biomedical research and the development of ^{3}H AMS would complement these studies. The ability to perform ^{3}H AMS measurements at sensitivities equivalent to those obtained for ^{14}C

will make it possible to perform experiments using compounds that are not readily available in ^{14}C-tagged form. In addition, unique double-labeling experiments could be performed in which the fate, distribution, and metabolism of separate fractions of biological compounds could be studied. For example, tritiated compounds could be utilized to establish DNA repair rates in conjunction with [^{14}C]PhIP exposures.

In years 1-2 of the grant we demonstrated that ^{3}H AMS can precisely and accurately measure ^{3}H in small biological samples. Within the last 2 years, we have used ^{3}H AMS to study two independent compounds, one ^{3}H-labeled and the other ^{14}C-labeled in the first low-level double-labeling experiment. The aim of this experiment was to demonstrate that two compounds could be studied separately within the a single animal, enabling us to investigate possible interactive effects of the compounds at doses relevant to human exposures.

The two compounds selected for this first study were [^{14}C]MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline) and [^{3}H]PhIP. MeIQx is a heterocyclic amine also formed during the cooking of meat, hence human exposure to both MeIQx and PhIP is likely to occur. Therefore, it is important to investigate if co-administration alters the pharmacokinetics or pharmacodynamics of the compounds by altering such things as absorption and/or distribution. Additionally, MeIQx and PhIP have different target organs for carcinogenicity in rodents (the target organ for MeIQx is liver while the target organs for PhIP are the colon and breast tissue), hence it is of interest to understand if differences in pharmacokinetics of these compounds may explain the differences in target organs.

In this experiment, we measured the levels of ^{3}H-labeled 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and ^{14}C-labeled 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in rat liver tissue and bound to liver DNA and protein 4.5 hours following acute administration of individual or co-administered doses in the range 4-5100 pmol/kg body-weight. Levels of PhIP and MeIQx in whole tissue and bound to liver protein were dose-dependent. MeIQx-protein and -DNA adduct levels were higher than PhIP adduct levels, which is consistent with their respective carcinogenicity in this organ. Co-administration of PhIP and MeIQx did not demonstrate any measurable synergistic effects compared to administration of these compounds individually.

A manuscript containing this data is currently in press in Chemical Research in Toxicology (Dingley *et al.*, 1998). In the future, this methodology can be used to look at the effects of 2 component mixtures in breast tissue.

CONCLUSIONS

During the period July 15, 1994-September 30, 1998 we have studied the pharmacokinetics of PhIP following acute oral and chronic administrations in accordance with specific aim 1. We have included both male and female F344 rats to provide useful comparisons between gender and to credibly assess our results versus published data performed with higher doses. In these studies we have shown that PhIP is bioavailable to the breast following oral administration and have been able to demonstrate dose-response trends for tissue concentrations of PhIP in liver, colon and breast tissue in both male and female F344 rats. In chronic studies we have

shown that PhIP accumulates in the tissues, including the breast and that excretion occurs mainly through the feces.

We have conducted studies to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups in accordance with specific aim 2. Additionally, we have investigated the effect of chlorophyllin treatment on the distribution of [^{14}C]PhIP. These studies have revealed that even at low human dietary equivalent doses, PhIP and PhIP metabolites are passed to sucklings pups and may pose a carcinogenic risk to the pups. Further, while chlorophyllin appears to be a reasonable detoxifying agent for the dams, it actually increases the exposure of the pups to PhIP.

In accordance with specific aim 3 we have established methodology for production and separation of oligonucleotides adducted with N-acetoxy PhIP. These adducts have been compared to *in vivo* adducts using ^{32}P-postlabeling. In addition, a portion of these polar adducts have ben characterized by triple-quadrapole mass spectrometry, UV absorbance and fluorescence spectroscopy. We have established that the majority of adducts formed *in vitro* are with deoxyguanosine and the major adduct both *in vitro* and *in vivo* is dG-C8 PhIP.

We have determined that PhIP forms DNA adducts at low dose in the breast and have shown the effect of dose of PhIP on PhIP-DNA adduct formation in female and male rodents in accordance with specific aim 4. Acute oral exposures to female and male F344 rats have been performed and linear dose-response relationships observed. Therefore, even at low dietary relevant doses, DNA adducts are formed in the breast and therefore may be involved in the carcinogenic effects of PhIP. Furthermore, we have shown that PhIP forms protein adducts in rats at low dose and that they might be useful markers of exposure and PhIP bioactivation for use in molecular epidemiology studies.

We have produced antibodies against PhIP-DNA in accordance with specific aim 5. This DNA has been used in a fluoroimmunoassy to detect and quantify PhIP-DNA adducts. Furthermore, tritium AMS has been successfully used to do the first low-level double-labeling experiment. This will enable us in the fuure to investigate the effects of 2 compound mixtures of chemicals which may be involved in the development of breast cancer.

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